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Highly efficient gene targeting in embryonic stem cells through homologous recombination with isogenic DNA constructs

(polymorphisms/retinoblastoma gene)

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ABSTRACT **A vast amount of data suggests that homologous recombination in mammalian cells is relatively rare as compared to random integration, imposing the need for sophisticated selection protocols to enrich for cells in which homologous recombination has occurred. We here show that one of the key factors in efficient homologous recombination is the use of isogenic DNA to prepare the targeting vectors. Homologous recombination at the retinoblastoma susceptibility gene (*Rb*) in embryonic stem cells derived from mouse strain 129 was 20-fold more efficient with a 129-derived targeting construct than with a BALB/c-derived construct. The two constructs were identical, except for a number of base sequence divergencies between 129 and BALB/c DNA, including base-pair substitutions, small deletions/insertions, and a polymorphic CA repeat. Transfection with an isogenic DNA construct, containing 17 kilobases of homology, yielded a targeting frequency of 78% (of a total of 20,000 drug-resistant colonies), without the use of an enrichment protocol for homologous recombination. This result indicates that, also in mammalian cells, homologous recombination rather than random integration can be the predominant event.**

Recently, it has become possible to inactivate or mutate specific genes in the mouse germ line (1). The procedure entails the introduction of the mutation through homologous recombination in embryonic stem (ES) cells, which, on fusion to recipient blastocysts, give rise to chimeric animals that may transmit the mutated allele to their offspring (2–5). Large variations in the number of homologous recombinants versus the total number of integration events (1/20 to 1/40,000) have been reported (4, 6–10). This has prompted the development of strategies to isolate cells that underwent homologous recombination from the large excess of cells that carry the DNA targeting construct integrated at random sites in their genome (11). Thus, PCR-based protocols allow the detection of homologous recombinants in pools of cell clones (12, 13). Selection procedures are aimed at the enrichment for the desired homologous recombination event by suppressing the formation of colonies carrying the targeting construct at random sites. In single selection protocols, targeting constructs contain a marker gene deprived of transcriptional and/or translational start signals in such a way that the juxtaposition of functional expression signals to the marker gene (conferring drug resistance to a cell) would be obtained on homologous recombination but only rarely on random integration (14–16). The double (or positive/negative) selection procedure developed by Capecchi and co-workers (7) makes use of an autonomously expressed marker gene, but the targeting construct is flanked by a second gene whose expression is detrimental to the cell. The flanking gene will be lost on homologous recombination but not on random integration, thus preventing the formation of drug-resistant colonies. The large variation in targeting efficiency for different genes cannot be attributed to differences in the size of the targeting constructs (10, 17) nor has any clear correlation between recombination efficiency and transcriptional activity of the target gene (18, 19), its chromosomal location (6), or other parameters been documented.

We hypothesized that the presence of base sequence divergencies between donor and recipient target DNA may reduce the efficiency of gene targeting. Such differences could arise when targeting vectors are constructed from a DNA library that originates from a different mouse strain than the ES cell line. Base-pair mismatches strongly affect the efficiency of genetic recombination in bacteria (20–22) and of intrachromosomal recombination in mammalian cells (23). We therefore compared the frequencies of homologous recombination at the retinoblastoma susceptibility gene in an ES cell line derived from mouse strain 129 with an isogenic (129-derived) and a nonisogenic (BALB/c-derived) DNA targeting construct. We report here on the large differences in recombination frequencies that were found and the base sequence divergencies seen between 129 and BALB/c DNA at the region of homology.

MATERIALS AND METHODS

Targeting Vectors. Genomic *Rb* fragments were obtained by screening BALB/c- and 129-derived genomic DNA libraries (the latter kindly provided by G. Grosveld) with a murine *Rb* cDNA probe (kindly provided by R. Bernards). *Rb* fragments were compared with human and murine *Rb* cDNA sequences (24, 25) and the partial human genomic *Rb* sequence (26) to determine the location of exons. *Neo*, *hyg*, and *Hprt* marker genes were inserted into the *Bgl* II site in the 19th exon of *Rb*.

Electroporation. ES cell line E14, derived from mouse strain 129 (kindly provided by M. Hooper) and its derivatives were cultured in BRL conditioned medium (27). Cells ($3–8 \times 10^7$) were mixed with 90 μg of targeting DNA in a volume of 600 μl of phosphate-buffered saline (PBS) and electroporated using a Bio-Rad Gene Pulser (0.8 kV, 3 μF; electrode distance, 0.4 cm). Cells were reseeded on 10-cm tissue culture dishes at a density of about 10^7 cells per plate. Drug selection (G418, 200 μg/ml; hygromycin B, 150 μg/ml; HAT medium, 0.1 mM hypoxanthine/0.8 μM aminopterin/20 μM thymidine) was started after 1 day; after 8–12 days, colonies were randomly picked and expanded.

DNA Analysis. DNA from individual drug-selected colonies was analyzed in the following way: $3–7 \times 10^5$ cells were embedded in 50 μl of 0.5% low-melting-point agarose in PBS and incubated in 1 ml of 0.5 M EDTA/1% sarkosyl/1 mg of proteinase K, pH 9.5, for 48 hr at 50°C. Agarose blocks were incubated three times for 1 hr at 50°C in 10 mM Tris/10 mM

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Abbreviations: ES, embryonic stem; nt, nucleotide(s).

EDTA, pH 8, plus 0.1 mM phenylmethylsulfonyl fluoride and once for 1 hr at room temperature in the appropriate restriction enzyme buffer. DNA digestion took place in 100 μl of restriction enzyme buffer containing 50 units of restriction enzyme for 6 hr at 37°C. Agarose blocks were melted at 65°C and loaded onto 0.7% agarose gels for Southern analysis.

Sequence Divergence. The *Rb* sequence present in targeting constructs 129Rb-neo and B/cRb-neo was digested into nine smaller fragments that were separated by low-melting-point gel electrophoresis. Each fragment was further digested with *Hin*fI or *Taq* I, ^{32}P-labeled with Klenow polymerase, and run on a denaturating polyacrylamide gel to detect restriction fragment length differences between the two constructs.

RESULTS

Rb Targeting with Isogenic and Nonisogenic DNA Constructs. DNA targeting constructs consisted of the neomycin phosphotransferase gene (*neo*), embedded in 10.5 kilobases (kb) of *Rb* sequence around the 19th and 20th exon of the gene (Fig. 1 *a* and *b*). The *Rb* sequence was isolated from either a mouse strain 129-derived genomic DNA library (construct 129Rb-neo) or a BALB/c-derived library (construct B/cRb-neo). The two constructs (Fig. 1*b*) were electroporated into ES cell line E14, which was derived from mouse strain 129 (27). Double crossing-over at the *Rb* locus will integrate the *neo* marker into the 19th exon of the *Rb* gene, thereby disrupting the coding sequence (Fig. 1*a*). G418-resistant colonies obtained from both electroporation experiments were analyzed by Southern hybridization. With B/cRb-neo, only one homologous recombinant was detected amid 144 random integration events. In contrast, of 94 G418-resistant colonies obtained with 129Rb-neo, 33 underwent homologous recombination at one of the *Rb* alleles. The DNA analysis of 40 of these colonies is shown in Fig. 2. In this experiment, gene targeting was 50-fold more efficient with 129Rb-neo than with B/cRb-neo. The isogenic targeting construct allowed the easy recovery of homologous recombinants (1 of 3 resistant colonies) without the use of any enrichment protocol.

Measuring Targeting Frequencies. To determine the difference in targeting efficiency of the isogenic and nonisogenic DNA constructs more accurately, a model system was designed that allowed the detection of even a low number of recombinants. First, the ES cell line E14TG2a, a hypoxanthine/guanine phosphoribosyltransferase (HPRT)-deficient derivative of E14 (27), was electroporated with targeting construct 129Rb-hprt, which contains an *Hprt* minigene embedded in 17 kb of a 129-derived *Rb* sequence (Fig. 1*c*). Cells were selected for the presence of the *Hprt* minigene in HAT-containing medium. Of 35 colonies that were analyzed, 8 contained the *Hprt* minigene, correctly integrated into the 19th exon of one of the *Rb* alleles through homologous recombination. None of the homologous recombinants contained additional *Hprt* copies integrated elsewhere in the genome (data not shown). One of these clones, designated HAT-20, was used as the recipient for a second targeting experiment using the constructs 129Rb-neo and B/cRb-neo. Double crossing-over at the previously targeted *Rb* allele will substitute *Hprt* for *neo*, giving colonies resistant to G418 (*neo*$^+$) and 6-thioguanine (*Hprt*$^-$). The ratio of G418-resistant, 6-thioguanine-resistant colonies to the total number of integrations (G418-resistant) obtained with 129Rb-neo was much larger than with B/cRb-neo (Table 1). However, 6-thioguanine-resistant colonies were also seen after electroporation of HAT-20 with the plasmid pMC1Neo poly(A), albeit at a much lower rate than with either targeting construct (Table 1). DNA analysis of these colonies revealed that all had lost the *Hprt*-containing *Rb* allele (not shown). Similar analyses demonstrated that 14 of 29 6-thioguanine-resistant

FIG. 2. Genetic modification of the *Rb* locus by homologous recombination with DNA targeting construct 129Rb-neo. DNA of individual G418-resistant clones was digested with *Eco*RI and analyzed by Southern hybridization using probe A (Fig. 1*a*). The nonmodified *Rb* locus gives a band of 9.7 kb (Rb); integration of *neo* by homologous recombination gives a 4.9-kb band (neo) (see Fig. 1 *a* and *b*).

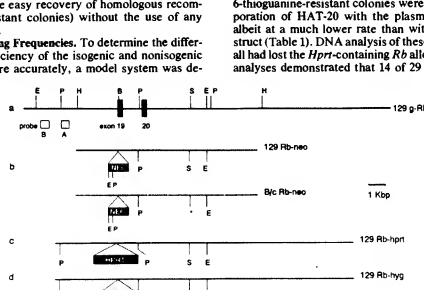

FIG. 1. DNA targeting constructs. (*a*) *Rb* locus around exons 19 and 20 (black boxes, numbered according to the human *Rb* gene organization; ref. 26; the location of other exons in this region was not determined) in mouse strain 129. B, *Bgl* II; E, *Eco*RI; H, *Hpa* I; P, *Pst* I; S, *Stu* I; The *Stu* I site was not present in the BALB/c sequence. A and B indicate probes to detect modifications at *Rb*. (*b*) DNA targeting constructs containing *neo* (from pMC1Neo poly(A); ref. 17; Stratagene) inserted into the *Bgl* II site of exon 19 within a 10.5-kb *Rb Hpa* I fragment derived from mouse strain 129 (129Rb-neo) or BALB/c (B/cRb-neo). A mutation in *neo*, reducing its ability to confer G418 resistance (28), was corrected. Two additional isogenic targeting constructs contained the *Hprt* minigene (from PGK*Hprt*; ref. 29) or the *hyg* gene (from PGK*hyg*; ref. 16) inserted into the *Bgl* II site of exon 19 within a 17-kb 129-derived *Rb* fragment, giving 129Rb-hprt (*c*) and 129Rb-hyg (*d*), respectively. These two constructs were flanked by nonendogeneous *Sal* I sites. All targeting constructs were separated from vector sequences by gel electrophoresis and purified by electroelution. Kbp, kilobase pair.

Table 1. Efficiency of homologous recombination

DNA	No. of HAT-20 cells	No. of colonies G418^R	G418^R, 6-TG^R	Ratio*
B/cRb-neo	5×10^7	11,500	105	1/200
129Rb-neo	5×10^7	13,500	1260	1/10
pMC1Neo poly(A)	2.5×10^7	5,470	11	—

Efficiency of homologous recombination at *Hprt*-containing *Rb* allele with isogenic and nonisogenic targeting constructs. ^{R}, Resistant; 6-TG, 6-thioguanine.
*Ratio of actual number of homologous recombinants (as deduced from Fig. 3) versus total number of G418^R colonies obtained.

colonies obtained with B/cRb-neo resulted from the spontaneous loss of the *Hprt*-containing allele rather than from homologous recombination, whereas all 6-thioguanine-resistant colonies obtained with 129Rb-neo were genuine homologous recombinants (Fig. 3 *A* and *B* show an example of this analysis). Corrected for the spontaneous loss of the *Hprt* minigene in the HAT-20 ES cell line, the frequency of homologous recombination at the *Hprt*-containing *Rb* locus was 1/10 for the isogenic targeting construct and 1/200 for the nonisogenic construct (Table 1). Thus, targeting at the *Rb* locus with isogenic DNA was 20-fold more efficient than with nonisogenic DNA.

DNA Polymorphisms Between 129 and BALB/c. The extent and the nature of the sequence divergence between the 10.5-kb *Rb* fragments present in the targeting constructs 129Rb-neo and B/cRb-neo were determined in the following way. First, the restriction digestion patterns of the two fragments were identical for 8 of 13 enzymes tested, showing that no gross alterations had occurred. Five restriction site polymorphisms were seen, suggesting that five base-pair substitutions were present within about 275 nucleotides (nt) that were tested in this way. Second, 1102 nt around the site where the *neo* marker was inserted and 585 nt 5 kb away from this site were sequenced. Within these two regions (in total, 1687 nt), nine base-pair substitutions, three small (1, 4, and 6 nt) deletions, and a polymorphic CA repeat (a 14-nt deletion) were detected in the BALB/c sequence with respect to the 129 sequence. The longest stretch of perfect homology within the sequenced region was 278 nt (Fig. 4 *Lower*). Finally, the remainder of the targeting constructs was examined for the presence of small deletions/insertions by restriction fragment length analysis on a denaturing polyacrylamide gel. Three additional deletions (2, 2, and 5 nt) and three insertions (1, 2, and 10 nt) were detected in the BALB/c

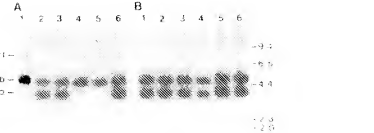

FIG. 3. Substitution of *Hprt* for *neo* at the *Rb* locus by homologous recombination in HAT-20 cells with targeting constructs B/cRb-neo (*A*) and 129Rb-neo (*B*). DNA of individual G418-resistant, 6-thioguanine-resistant clones was digested with *Pst* I and analyzed by Southern hybridization, using probe A (Fig. 1*a*). The positions of bands corresponding to the wild-type *Rb* allele (4.9 kb, Rb), the *Hprt*-containing *Rb* allele (7.7 kb, hprt), and the integration of *neo* (3.7 kb, neo) are indicated (compare Fig. 1 *b* and *c*). Homologous recombination is seen in lanes 2, 3, and 6 of *A* and lanes 1–6 of *B*. Lanes 1, 4 and 5 of *A* show the spontaneous loss of the *Hprt*-containing *Rb* allele.

fragment with respect to the 129 fragment (Fig. 4 *Upper*). Based on these results, we estimate that, on the average, one sequence difference (a base-pair substitution or a deletion/insertion) was present per 160 nt.

Double Knockout of Rb. The improved efficiency of "perfect match" DNA constructs in gene targeting is clearly demonstrated by an experiment in which both *Rb* alleles were disrupted by two consecutive rounds of homologous recombination. First, one *Rb* allele was disrupted by homologous recombination with a BALB/c-derived targeting construct, containing ≈18 kb of *Rb* sequence. By employing the positive/negative selection strategy, three correct integrations of a *neo* marker into the 19th exon of the *Rb* gene were isolated amid 3600 random integration events (result not shown). One of these single *Rb* knockout cell lines was used as the recipient in a second electroporation experiment with an isogenic targeting construct, consisting of a hygromycin-resistance gene embedded in 17 kb of a 129-derived *Rb* sequence (129Rb-hyg, Fig. 1*d*). After selection for hygromycin B resistance, 20,000 colonies were obtained, of which 78% (48 of 61 tested colonies) resulted from homologous recombination. Clearly, in this experiment, homologous recombination rather than random integration was the predominant event. In the majority of targeting events (40 of 48), the *hyg* gene was integrated in the wild-type Rb allele, giving cell

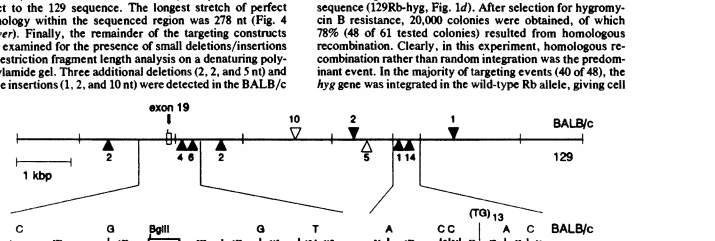

FIG. 4. Sequence divergencies between BALB/c and 129 DNA at the region of homology. (*Upper*) The 10.5-kb *Rb* sequence, present in targeting constructs B/cRb-neo and 129Rb-neo (Fig. 1*b*). The sequence is divided into nine smaller fragments, separated by vertical bars. Filled triangles represent extra nucleotides in the BALB/c sequence (above the line) or the 129 sequence (below the line). Open triangles indicate length differences within a fragment that could also result from restriction site polymorphisms. (*Lower*) Nucleotide differences determined by sequence analysis of the indicated regions.

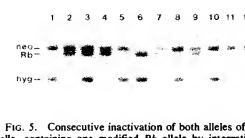

FIG. 5. Consecutive inactivation of both alleles of *Rb*. E14 ES cells, containing one modified *Rb* allele by integration of a *neo* marker into the 19th exon, were electroporated with targeting construct 129Rb-hyg (Fig. 1*d*). DNA of individual hygromycin B-resistant colonies was digested with *Eco*RI and analyzed by Southern hybridization, using probe B (Fig. 1*a*). The positions of bands corresponding to the nonmodified *Rb* allele (9.7 kb, Rb), the integration of *neo* (11.5 kb, neo; note that this *neo* marker did not contain an *Eco*RI site), and the integration of *hyg* (4.9 kb, hyg) are indicated. Double knockouts are seen in lanes 1, 3, 5, 8, 10, 11, and 12; in lane 3, a nonmodified *Rb* copy is still present, suggesting that this clone was trisomic for the *Rb* locus or that flanking sequences had been transferred from the endogenous *Rb* gene to the targeting construct, which then integrated elsewhere (8, 16); in lane 6, *hyg* has replaced *neo*; in lanes 2, 4, 7, and 9, no targeting has occurred by *hyg*.

lines in which both *Rb* alleles were disrupted. In the remaining eight recombinants, the *hyg* sequence had replaced *neo* (Fig. 5 shows an example of the DNA analysis).

DISCUSSION

Sequence Divergence Among Laboratory Mice. Our results demonstrate that allelic differences at the *Rb* locus between different mouse strains imposed a major handicap in mutating the gene through homologous recombination. Although we have not performed a similar detailed analysis with other genes, recent experiments in our institute have shown that targeting to the multidrug-resistance gene *mdr-1b* could be obtained with a frequency of 15%, using a 129-derived construct, without the application of any enrichment protocol for homologous recombination (A. Schinkel, J. Smit, and P. Borst, personal communication). Could the use of nonisogenic DNA targeting constructs have played an important role in the large variation in targeting efficiencies that have been reported during the past years? Our results suggest that the efficiency of gene targeting with nonisogenic DNA constructs is influenced by the extent of allelic differences of the target gene. Variation in allelic differences for different genes may find its origin in the derivation of the current laboratory mouse strains. Many of these strains (including BALB/c and 129) carry a well-conserved Y chromosome, apparently derived from a common ancestor (30). Laboratory mouse strains are therefore regarded as genetic hybrids (30). They carry identical (or nearly identical) alleles inherited from a common ancestor *and* diverged alleles inherited from genetically distant ancestors (31). Depending on the distribution pattern of different alleles of a given gene over different mouse strains, homologous recombination with nonisogenic DNA constructs may be more or less efficient.

Prevalence of Homologous Recombination. It is generally believed that homologous recombination in mammalian cells is relatively rare as compared to random integration. However, in our targeting experiments homologous recombination frequencies of up to 80% could be reached, indicating the prevalence of homologous recombination over random integration in mammalian cells. Homologous recombination was only rarely accompanied by random integration in the same cell (as was also reported earlier; ref. 32). Thus, efficient gene targeting resulted in cell clones carrying the marker gene exclusively integrated at the target locus.

Effect of Polymorphisms on Recombination. The sequence divergence between BALB/c and 129 at the *Rb* locus was sufficient to drastically reduce homologous recombination in 129-derived ES cells with the BALB/c-derived targeting construct. In studies of intrachromosomal recombination in mammalian cells, the rate of homologous recombination appeared to be determined by the presence of several hundred base pairs of uninterrupted homology rather than by the total number of mismatches (33, 34). We found that in a region of 1687 nt sequenced within the BALB/c-derived *Rb* targeting construct, the longest stretch of perfect homology with the 129 target locus was only 278 nt, suggesting that the lack of sufficiently long, perfectly matched regions impaired efficient targeting. However, base-pair mismatches may dominantly suppress homologous recombination as well. In several bacterial systems (*Escherichia coli*, ref. 22; *Streptococcus pneumoniae*, ref. 20), particular base-pair mismatches in heteroduplex regions formed at an initial stage of recombination trigger long-patch mismatch repair leading to dissociation of the mismatch-containing heteroduplex and abortion of the recombination reaction. Indeed, there is some evidence for the existence of long-patch mismatch repair in yeast (35) and in higher eukaryotic cells (36, 37), although its effect on homeologous (involving not perfectly matched DNA partners) recombination is not known.

Other Factors Affecting Homologous Recombination. In the course of our experiments, we found that the use of a mutated version of the *neo* gene reduced the recovery of homologous recombinants more than the recovery of random integrants. This observation suggests that the expression level of the mutant marker gene at the *Rb* locus is around or even below the threshold required for drug resistance. Therefore, homologous recombination at some target loci may remain undetected due to insufficient expression of the marker gene at that site.

Although we consider the use of perfect match targeting constructs an important requirement for efficient gene disruption in mammalian cells, other factors may affect the rate of homologous recombination as well. Several of these parameters (e.g., transcriptional activity and chromosomal location of the target gene, nature of the mutation, delivery of DNA, composition of targeting construct) may need reconsideration within the context of our results. In addition, the unraveling of the enzymatic machinery that catalyzes the matching of homologous sequences and the exchange of genetic information could provide a further boost in our capacity to efficiently modify specific genes in eukaryotic cells.

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EXHIBIT A